Week 10

August 8, 2016

What We Did Today:
Digested pgRNA with BstX1 and xho1 for Gibson Vector

Sequencing

pick 4 colonies of WT and PTC colonies from each Gibson plate

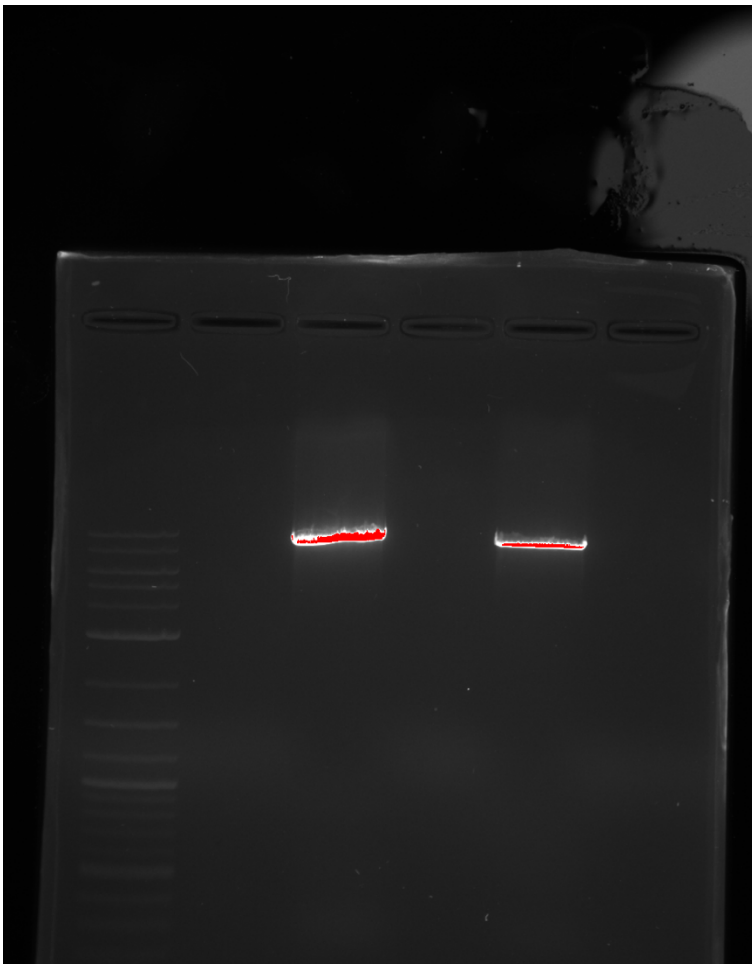
• Split cell culture
Confluence: >90%
1:5 split
• Cell plating
Cell count: 339; 371 → Average = 355
• $1.42 * 10^6$ cells/mL
Plating:
12-well plate: $3*10^5$ cells * 13 wells → 2.75 mL cells

Purified pSB1C3

August 9, 2016

What We Did Today:

Gel purified digest from overnight
Prepped art for A term



Gel – pgRNA Vector

Lane 1 –ladder
Lane 2 – Empty
Lane 3 – 30ul Sample
Lane 4 – Empty
Lane 5 – 30ul Sample

• Transfection

APOBEC trial 2

- Row 1: pRetro empty
- Row 2: pRetro-APO2X-dCas9
- Row 3: pRetro-APO3X-dCas9

• Cell plating

Cell count: 127; 112 → Average = 120

- 0.48 * 10^6 cells/mL

Plating:

12-well plate: 3*10^5 cells * 13 wells → 8.125 mL cells

Miniprep

Digest with Not1 to linearize
neg control GFPn3
16uL water, 1uL each enzyme, 2uL buffer
gel:
L, neg, WT1-4, PTC1-4

Test digested biobrick ATG/ACG eGFP, run on gel

August 10, 2016

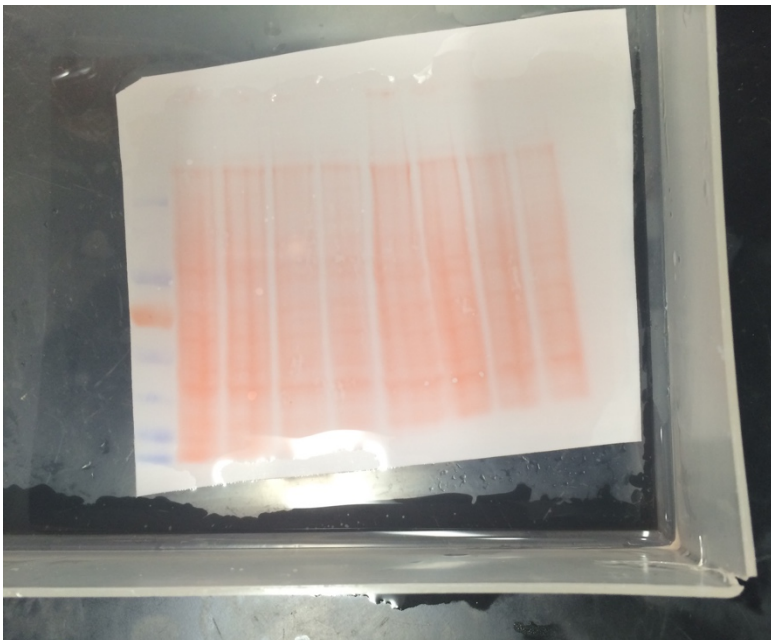
What We Did Today:

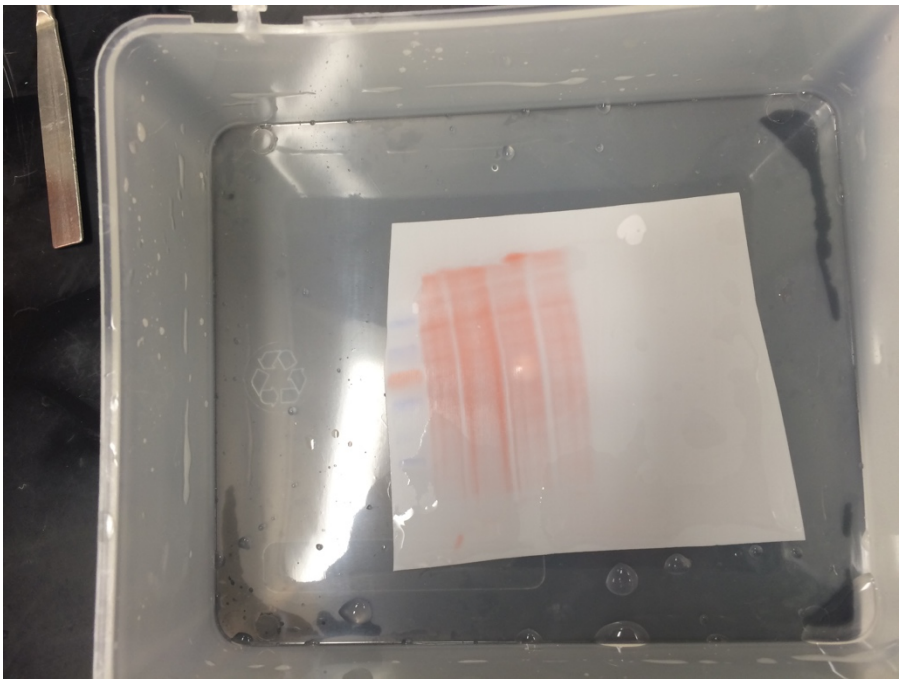
Western Blot
Gibson gRNA

Western Blot Protocol

Used premade gels
15 µL of each sample added to each well
Ran for an hour and a half on 120 V
Took gel out of plastic container and stored in water
In a Pyrex dish containing **Transfer Liquid,** made stacks using two sided clasp, sponge, filter paper, gel, transfer paper, filter paper, sponge and closed clasp, being careful to keep it always wet with transfer liquid and without air bubbles.
Put in blot box filled with transfer liquid, stir bar, and ice box. Ran at 100 V for 1 hour.
Removed from blot box, took out the membrane and put in water, added **Ponceau S Stain** liquid, drained liquid, rinsed with water and photographed.
Rinsed with **Tween Wash Buffer** until clean.

Moved membrane into bag, added 10 mL **Dry milk and Tween PBS wash buffe**r.
Removed bubbles and sealed bag. Refrigerated overnight.





Transfer Liquid

50 ml 10/20x
100 ml meOH
850 water

Ponceau S Stain liquid

95 ml water added to 0.1g Ponceau S Power
5 ml glacial acetic acid added last

Tween Wash Buffer

100 ml 10x PBS
900 water
5 ml 20% Tween 20

Dry milk and Tween PBS wash buffer

2g dry nonfat milk
40 ml Tween Wash Buffer

Gibson Protocol

Scrambled pgRNA

9ul pgRNA vector
1ul Insert
10ul Gibson mix

ACG-GFP pgRNA

9ul pgRNA vector
1ul Insert

10 ul Gibson mix

Globin pgRNA
9ul pgRNA vector
1 ul Insert
10ul Gibson mix

Negative Control
9ul pgRNA vector
10ul Gibson mix
1ul Water

• Induction
Doxycycline → APOBEC plate
- Column 1: 0
- Column 2: 10 ng/mL
- Column 3: 25 ng/mL
- Column 4: 50 ng/mL

Transfected globin into cells
Sequenced WT/PTC
Rerun ACG eGFP biobrick samples on gel to confirm

August 11, 2016

Gibson Plate Counts/Liquid Cultures

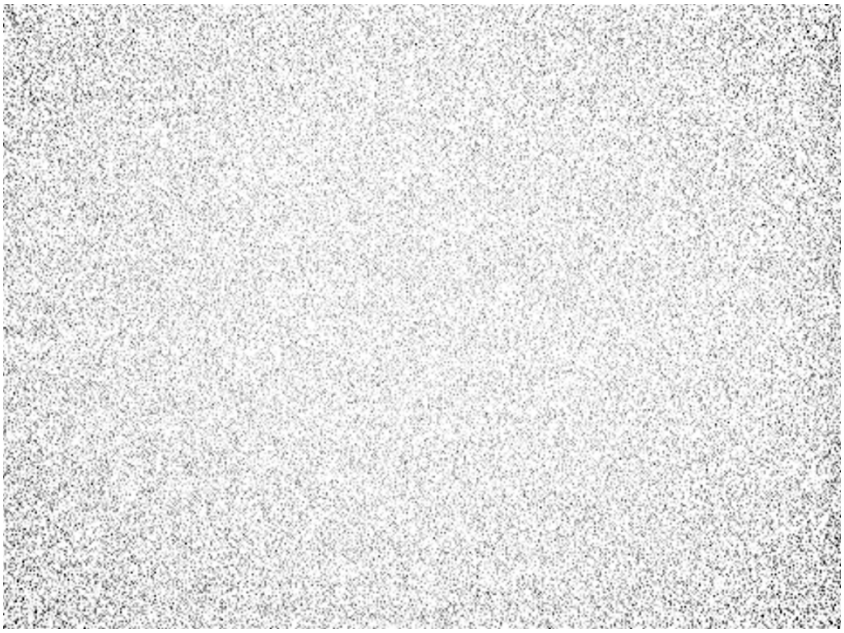
Neg. Control #1,2,3 – 0
Scrambled pgRNA
- #1 – 1
- #2 – 4
- #3 – 0

Globin #1,2,3 – 0
ACG-GFP pgRNA
- #1 – 0
- #2 – 1
- #3 – 1

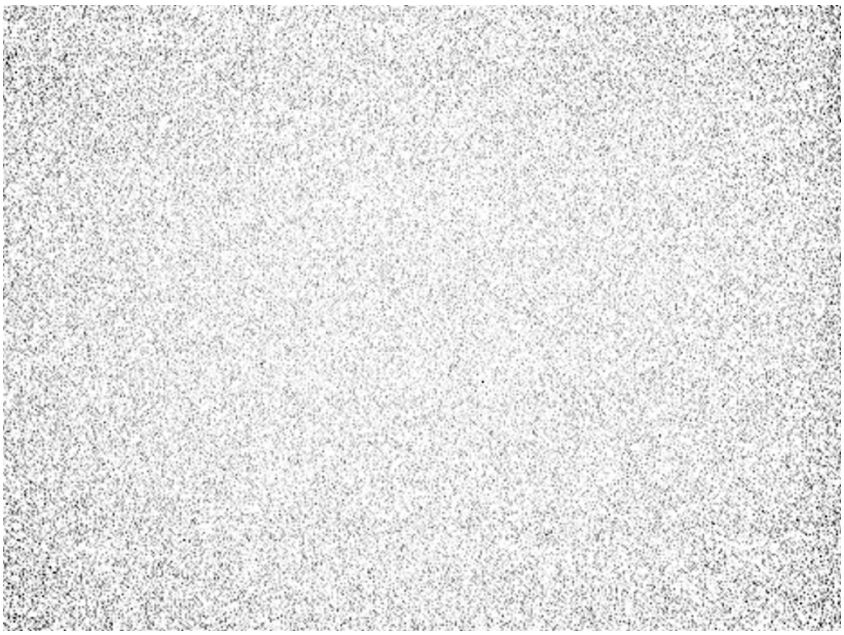
Western Blot

Took blots out of overnight milk
Added 15 mL milk and 1.5 uL mono-clonal anti-flag
Shook for 1 hour
Rinsed with wash buffer and shook for 10 mins (3x)
In a tube mixed 1 mL of each developer and add to blot
Shook for 5 minutes
Placed between two layers of plastic wrap
Measured on imaging machine using Chemi protocol at different exposures

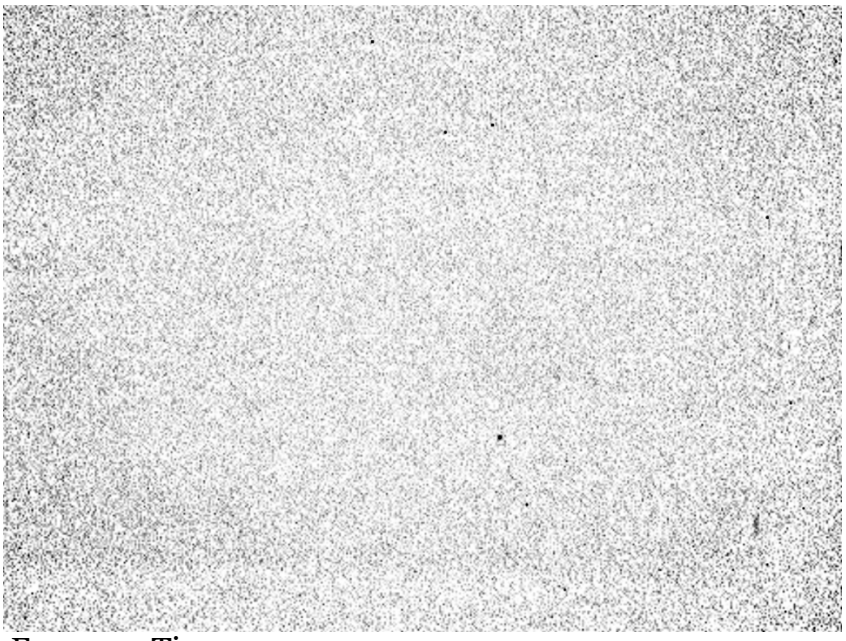
Blot 1



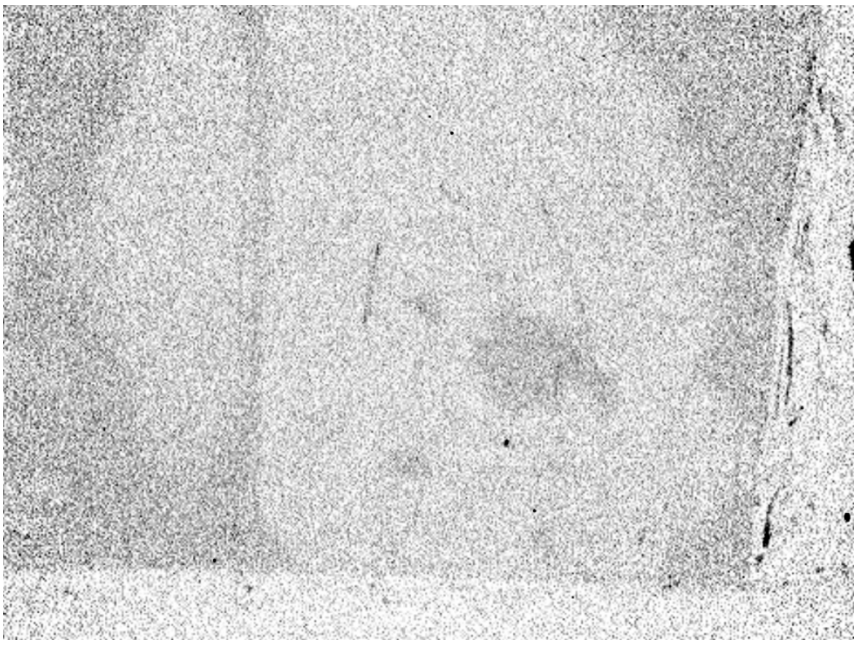
Exposure Time: 0.38 secs



Exposure Time: 2 secs

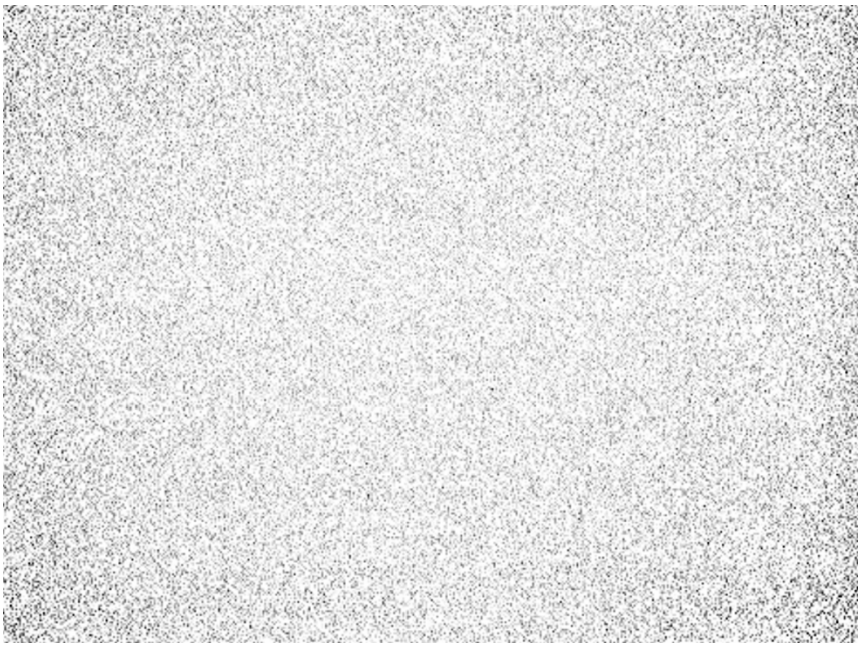


Exposure Time: 45 secs

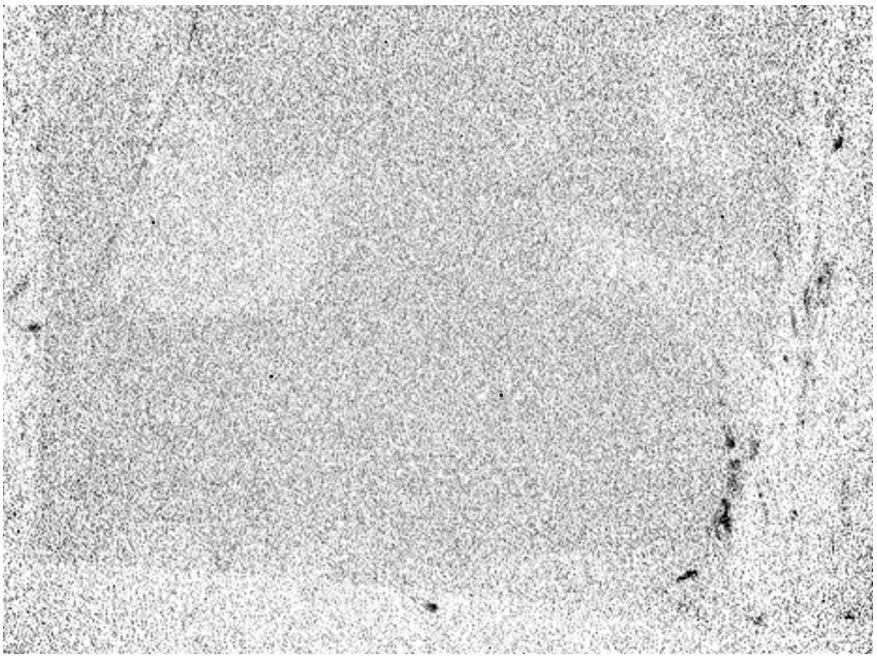


Exposure Time: 60 secs

Blot 2

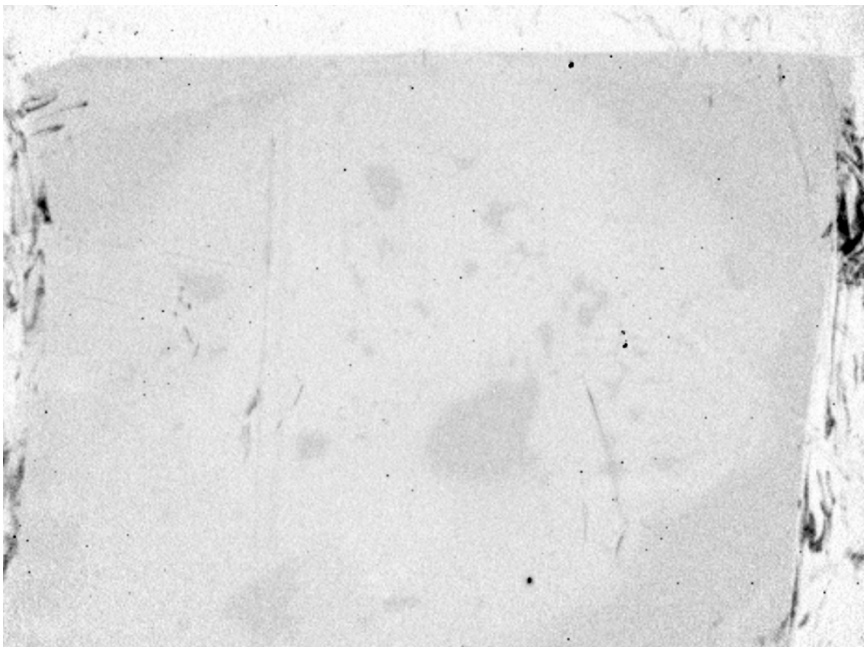


Exposure Time: 2 secs



Exposure Time: 10 secs

Exposure Time: 60 secs



Exposure Time: 10 mins

Gibson Cloning Globin pgRNA

Mixture
- 9ul Vector
- 2 ul Insert
- 10 ul Gibson mix

Took pictures
Miniprepped more pgRNA
Set up+ sent off Biobrick ACG+ATG eGFP plasmids for sequencing

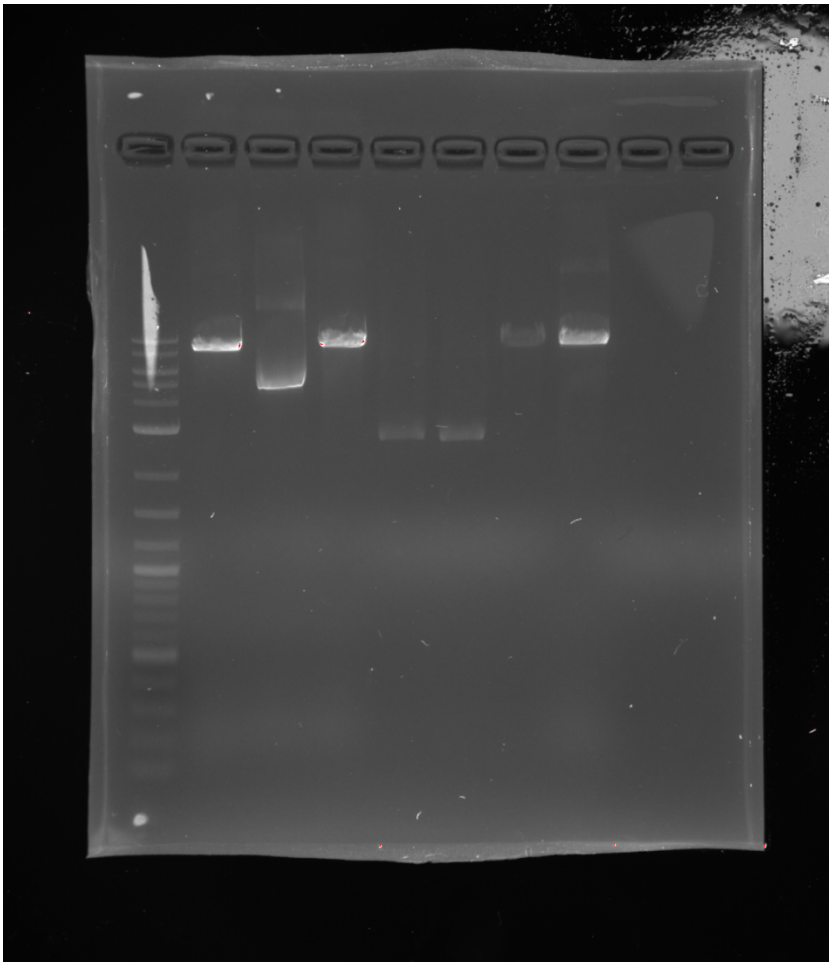
• Harvest APOBEC plate (for Western blot)
- 1.5 mL 2xSDS sample buffer + 30 µL DTT
- Heat at 80 ˚C, 10 min
- Store in freezer

August 12, 2016

Gibson Globin Counts/Liquid Cultures

Neg. Control #1,2,3 – 0
Globin
- #1 – 0
- #2 – 0
- #2 – 1

Miniprepped
Digested Scrambled and ACG-GFP pgRNAs with BstX1 and xHo1
Ran on Gel



Lane 1 – Ladder
Lane 2 – Neg. Control
Lane 3 – Scrambled #1
Lane 4 – Scrambled #2
Lane 5 – Scrambled #3
Lane 6 – Scrambled #4
Lane 7 – ACG-GFP #1
Lane 8 – ACG-GFP #2

Analysis
The Scrambled #1 and ACG-GFP #2 looked like they worked because the insert is 150bp and these are slightly bigger than the negative control